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Purification and cellular localization of wild type and mutated dihydrolipoyltransacetylases from *Azotobacter vinelandii* and *Escherichia coli* expressed in *E. coli*

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Wild type dihydrolipoyltransacetylase(E2p)-components from the pyruvate dehydrogenase complex of *A. vinelandii* or *E. coli*, and mutants of *A. vinelandii* E2p with stepwise deletions of the lipoyl domains or the alanine- and proline-rich region between the binding and the catalytic domain have been overexpressed in *E. coli* TG2. The high expression of *A. vinelandii* wild type E2p (20% of cellular protein) and of a mutant enzyme with two lipoyl domains changed the properties of the inner bacterial membrane. This resulted in a solubilization of *A. vinelandii* E2p after degradation of the outer membrane by lysozyme without any contamination by *E. coli* pyruvate dehydrogenase complex (PDC) or other high-molecular-weight contaminants. The same effect could be detected for *A. vinelandii* E2o, an E2 which contains only one lipoyl domain, whereas almost no solubilization of *A. vinelandii* E2p with one lipoyl domain or of E2p consisting only of the binding and catalytic domain was found. Partial or complete deletion of the alanine- and proline-rich sequence between the binding and the catalytic domain did also decrease the solubilization of the E2p-mutants after lysozyme treatment. Immunocytochemical experiments on *E. coli* TG2 cells expressing *A. vinelandii* wild type E2p indicated that the enzyme was present as a soluble protein in the cytoplasm. In contrast, overexpressed *A. vinelandii* E2p with deletion of all three lipoyl domains and *E. coli* wild type E2p aggregated intracellularly. The solubilization by lysozyme is therefore ascribed to excluded volume effects leading to changes in the properties of the inner bacterial membrane.

Introduction

The pyruvate dehydrogenase complex, which catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA, is built from multiple copies of three enzymes: pyruvate dehydrogenase (E1p), dihydrolipoyltransacetylase (E2p) as the core component and lipoamide dehydrogenase (E3) [1]. Dihydrolipoyltranssuccinylase (E2o) is the corresponding core enzyme in the 2-oxoglutarate dehydrogenase complex [2].

The structure of E2p from *Azotobacter vinelandii* and *Escherichia coli* is formed by five domains (Fig. 1) [3–5]. The N-terminal part, called lipoyl domain, consists of three homologous repeating sequences [3,4], forming lipoyl subdomains, which are highly charged. Each lipoyl subdomain contains a lysyl residue as a potential covalent attachment site for a lipoyl moiety. The catalytic domain is located on the C-terminal part and contains the acetyltransferase active site and the polymer interface. A region of around 60 amino acid residues, which is flanked by proteinase sensitive sites, is located between the two larger domains. In E2p from *A. vinelandii*, E1p [6] and E3 [7,8] interact with this region (binding domain). Site-directed mutagenesis experiments have also indicated residues on the N-terminus of the catalytic domain that are involved in E1p-binding [7]. The E2p-domains are linked to each other by segments rich in alanine and proline. These segments are somewhat extended in their structure as well as conformationally mobile [9]. By that way they keep the E2-domains separated and allow highly flexible movements, which are the basis of the catalytic mechanism within the complex [10]. The catalytic domain forms a highly symmetric cubic structure from which lipoyl and binding domain are protruding (Refs. 2 and 5, A. Mattevi, personal communication). The lipoyl and catalytic domain from *A. vinelandii* E2p can be isolated individually after proteolytic digestion [11], resulting in loss of binding of the peripheral compo-

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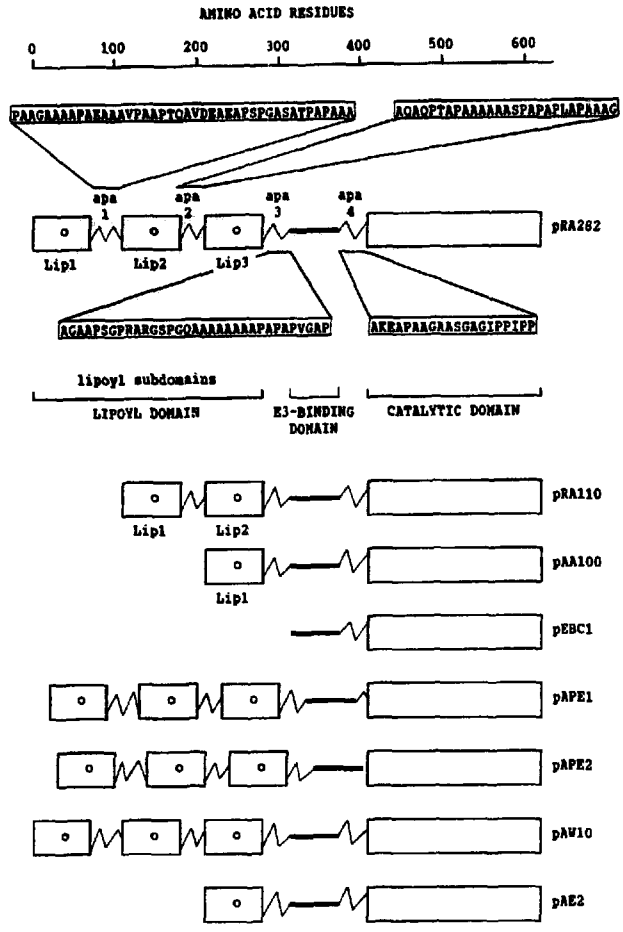

Fig. 1. Domain structure of wild type E2p (pRA282) and E2o (pAE2) from *A. vinelandii*, wild type E2p from *E. coli* (pAW10) and of the *A. vinelandii* E2p-mutants with two lipoyl domains (pRA110), one lipoyl domain (pAA100) and complete deletion of all three lipoyl domains (pEBC1). In the E2p-mutants encoded by the plasmids pAPE1 and pAPE2 the APA-region between the binding domain and the catalytic domain (APA-4) is partially or totally deleted. The E2-genes were subcloned in pUC9, resulting in the plasmids given above. The lipoylation site within the lipoyl domains is indicated by an open circle (o). Tilted lines represent parts of the E2-chain rich in alanine, proline and charged amino acids (APA 1–4). The primary structures of these interdomain segments are shown in exploded view for *A. vinelandii* wild type E2p.

nents. Binding of both peripheral components to the binding domain was also described for E2p from *Bacillus stearothermophilus* [12] and for the E2b-component of the branched chain α-ketoacid dehydrogenase complex from bovine liver [13]. On the other hand, limited proteolysis of *E. coli* dihydrolipoyltranssuccinylase (E2o) resulted in loss of the E3-binding, whereas E1o was still bound to the catalytic domain [14].

Upon binding of E1p and E3, the E2p from *A. vinelandii* behaves in a unique way, because its core structure of 24 subunits dissociates into smaller structures [15,16]. E2p from *E. coli* does not dissociate upon binding of E1p and/or E3 [9]. Therefore the *E. coli* PDC is much larger than that of *A. vinelandii*. The domain structure of E2o (*A. vinelandii* and *E. coli*) differs from E2p in having only one lipoyl domain [17,18]. Upon binding of peripheral components, E2o from *A. vinelandii* is also not dissociating.

The genes for the dihydrolipoyltransacetylase from *E. coli* [4] and *A. vinelandii* [3] have been cloned and sequenced. Overexpression and purification of wild type E2p and of the catalytic domain from *A. vinelandii* have been reported [8,11].

In this paper we describe the expression of wild type E2p and of E2p-mutants from *A. vinelandii* and of wild type E2p from *E. coli* in *E. coli* TG2 cells. The influence of lysozyme on the solubilization of the different E2p-mutants expressed in *E. coli* TG2 cells was studied. Immunocytochemical experiments were performed on *E. coli* TG2 cells expressing dihydrolipoyltransacetylases from *A. vinelandii* and *E. coli* in order to examine the intracellular localization of the E2p-proteins and the influence of the synthesized E2p on the properties of the bacterial cell membrane. This study offers a rationale for the purification of mutant enzymes obtained by whole domain deletions of E2p-proteins from *A. vinelandii* and *E. coli* and of chimeric proteins, constituted from domains derived from E2p of both organisms.

Materials and Methods

Materials

Restriction endonucleases, DNA polymerase I (Klenow fragment) and T4-DNA-ligase were purchased from Bethesda Research Laboratories. Lysozyme, calf intestinal phosphatase and universal M13 sequencing primer were obtained from Boehringer. [α-^{32}P]dATP (3000 Ci/mmol) was purchased from New England Nuclear. DEAE-Sephacel and Sephacryl S-400 were from Pharmacia Fine Chemicals. All other chemicals used were of analytical grade.

Bacterial strains and vectors

E. coli TG2, a *recA*$^-$ version of TG1 [Δ(*lac-pro*), *thi*, *supE*, [Res$^-$ Mod$^-$ (k)], F′(*traD36 proA*$^+$*B*$^+$, *lacI*^{q}Z ΔM15)] [19] was used as a host for the expression of the plasmid-encoded wild type E2p or the E2p-mutants. The plasmid pUC9 [20] was used for subcloning and the phage M13mp18 [21] for sequencing. Standard DNA operations were performed as described in Ref. 22.

Construction of mutant enzymes

The domain structure of *A. vinelandii* wild type E2p and E2o, of the E2p-mutants and of *E. coli* wild type E2p is described in Fig. 1. The alanine- and proline-rich sequences between the last lipoyl domain and the binding domain (APA-3) as well as between binding and catalytic domain (APA-4) of the E2p-proteins are given in Fig. 2.

Plasmid	APA-3 (Lipoyl - binding domain)	APA-4 (Binding - catalytic domain)
pRA282	AGAAPSGPRARGSPGQAAAAAAAAPAPAPVGAP	AKEAPAAGAASGAGIPPIPP
pRA110	GAQAQPTAPAAAAAPAPAPVGAP (APA-2)	AKEAPAAGAASGAGIPPIPP
pAA100	PAAGAAAAAPAPAPVGAP (APA-1)	AKEAPAAGAASGAGIPPIPP
pEBC1	----------	AKEAPAAGAASGAGIPPIPP
pAPE1	AGAAPSGPRARGSPGQAAAAAAAAPAPAPVGAP	AK----------GIPPIPP
pAPE2	AGAAPSGPRARGSPGQAAAAAAAAPAPAPVGAP	A----------
pAW10	AAPAAAPAKQEAAAPAPAAKAEAPAAAPAAKA	AEAAPAA
pAE2	ATAAPAAAPAPAAAAPAAAEAP	AAAEAKKSAPAGQPAPAATAAPLPAA

Fig. 2. Interdomain segments between the last lipoyl subdomain and the binding domain (APA-3) and between the binding and the catalytic domain (APA-4). The primary structures of the APA-regions are given for *A. vinelandii* wild type E2p (pRA282), E2p with two lipoyl domains (pRA110), E2p with one lipoyl domain (pAA100), E2p binding plus catalytic domain (pEBC1), E2p with partial (pAPE1) or complete (pAPE2) deletion of APA-4, *E. coli* wild type E2p (pAW10) and *A. vinelandii* wild type E2o (pAE2). The underlined amino acids of APA-3 for pRA110 and pAA100 indicate residues, which are in the wild type E2p parts of APA-1 or APA-2, respectively.

For the construction of the *A. vinelandii* E2p-mutants the plasmid pRA282 (5.4 kB) [3], containing the complete E2p-gene in pUC9, was used as starting material. A partial restriction map of this plasmid is shown in Fig. 3. The nucleotide sequence in the APA-3 region (base pairs 1400–1414 in the original paper [3]) was corrected after new sequencing informations to GCTGCGGCCGCTGCC (changed bases are underlined), resulting in the amino acid sequence AAAAA (residues 316–319).

For the construction of an E2p-mutant with two lipoyl subdomains the plasmid pRA282 was digested by *Not*I and the plasmid pRA110 was obtained after ligation of a resulting 5.1 kB *Not*I-fragment. In this plasmid the third lipoyl subdomain is deleted and the APA-3 region is shortened from 33 to 23 residues by a fusion of 11 residues from APA-2 with 12 residues from APA-3 (Figs. 1 and 2).

The plasmid pAA100 with one lipoyl subdomain was constructed after digestion of pRA110 by *Sst*II and ligation of the resulting 4.7 kB-fragment, in which the second and the third lipoyl subdomain are deleted. The

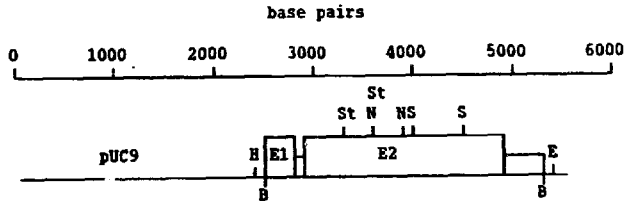

Fig. 3. Partial restriction map of the plasmid pRA282, containing the C-terminus of the E1-gene and the complete E2p-gene from *A. vinelandii*. Restriction sites are shown for *Hin*dIII (H), *Bam*HI (B), *Sst*I (St), *Not*I (N), *Sph*I (S) and *Eco*RI (E).

APA-region between lipoyl and binding domain is shortened to 18 residues and consists of 6 amino acids from APA-1 and 12 residues from APA-3.

For the construction of the plasmid pEBC1, containing only the binding domain and the catalytic domain of *A. vinelandii* E2p, pRA282 was digested by *Hin*dIII. The *Hin*dIII-site was filled using the Klenow-fragment of DNA-polymerase I. Next the DNA was partially digested by *Sph*I and a 4.2 kB-fragment was isolated. The *Sph*I-site was made blunt-end by T4-polymerase. After ligation and transformation into *E. coli* TG2 a fusion protein consisting of the first five amino acids from β-galactosidase, the binding domain of E2p starting with residue number 339 and the catalytic domain could be produced. This protein contains only one APA-sequence (APA-4).

The plasmids pAPE1 and pAPE2 in which the APA-region between the binding domain and the catalytic domain (APA-4) is partially or totally deleted are described in Ref. 23.

The plasmid pAE2, encoding the dihydrolipoyltranssuccinylase (E2o) of the 2-oxoglutarate dehydrogenase complex from *A. vinelandii* is described in Ref. 17.

Cloning of the genes coding for E2p and E3 from E. coli

Cloning and sequencing of the genes coding for the dihydrolipoyltransacetylase (E2p) and the lipoamide dehydrogenase (E3) from *E. coli* has been described [4,24]. For our investigation, chromosomal DNA of *E. coli* K12 was digested with *Eco*RI, because it was known that this enzyme cuts in front of the gene coding for the E2p but not within the E2p- and E3-genes [4,24]. The digest was size fractionated on an agarose gel and parts of the gel containing DNA-fragments of 5–10 kB were cut out. Next these fragments were ligated into the *Eco*RI-site of pUC9. *E. coli* TG2 cells, transformed with recombinant plasmids, were grown on two-gridded nitrocellulose filters, placed on TY-agar plates, lysed according to Ref. 25, and screened for the production of *E. coli* lipoamide dehydrogenase, using antiserum against *E. coli* E3. One positive plasmid (pAW9) containing an 8.9 kB *Eco*RI-insert was found. *E. coli* TG2 cells transformed with this plasmid produced high amounts of lipoamide dehydrogenase and after induction with isopropylthio-β-D-galactoside (IPTG) also large amounts of dihydrolipoyltransacetylase, comparable to that found for the cloned E2p and E3 from *A. vinelandii* [3,25]. The E2p-gene was then subcloned, yielding a plasmid (pAW10) with a 3.2 kB *Kpn*I-*Dra*I insert containing the complete gene. *E. coli* cells transformed with this plasmid also showed after induction with IPTG a high expression of the dihydrolipoyltransacetylase component.

Preparation of cell free extracts

E. coli TG2 cells, harbouring recombinant plasmids, were grown at 37°C in TY-medium, containing ampicillin (75 μg/ml) and IPTG (20 μg/ml) for at least 20 h, unless described otherwise. 10 ml of the cell culture were centrifuged for 10 min at 6000 × *g*. The cell pellet was suspended in 1 ml TEG-buffer (25 mM Tris-HCl (pH 8.0), 50 mM glucose, 10 mM EDTA). Lysozyme (10 μl, 50 mg/ml) was added to 500 μl of this suspension and incubated for 45 min at 37°C. The supernatant after centrifugation at 11000 × *g* for 10 min was called lysozyme fraction (L). The other 500 μl from the cell suspension were directly sonicated (30 W, 3 mm tip-diameter, 3 × 10 s) and the supernatant after centrifugation at 11000 × *g* for 10 min was called sonicated fraction (*S*).

Assays

The E2p-activity was assayed spectrophotometrically at 240 nm as described previously [26]. The overall activity of the pyruvate dehydrogenase complex was measured as described in Ref. 26.

The activity of isocitrate dehydrogenase was assayed in 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM $NADP^+$, 50 mM isocitrate and 3 mM $MgCl_2$. For the estimation of the activity of alkaline phosphatase 200 μl cell free extract were mixed with 700 μl destilled water and 100 μl 27 mM *p*-nitrophenylphosphate in 1 M Tris-HCl (pH 9.7). After 30 min at 37°C the reaction was stopped by adding 150 μl 6 M NaOH and the extinction was measured at 420 nm. 1 unit catalyzes the conversion of 1 μmol 4-nitrophenylphosphate per min under test conditions.

Protein concentrations were estimated with the microbiuret method [27] after a precipitation with deoxycholic acid and trichloracetic acid [28]. Bovine serum albumin was used as a standard.

Isolation of A. vinelandii E2p and E. coli E2p expressed in E. coli TG2

Wild type E2p from *A. vinelandii* and the E2p-mutant with two lipoyl subdomains (pRA110) were isolated from *E. coli* TG2 as described in Ref. 11 with the following modifications: 25–30 g cells from a 6 l culture were suspended in 150 ml 50 mM potassium phosphate buffer (pH 7.0), containing 3 mM EDTA and 0.1 mM phenylmethylsulphonyl fluoride ($PhMeSO_2F$) (standard buffer). After the addition of 70 mg lysozyme the suspension was incubated for 2 h at 37°C. After centrifugation for 30 min at 14000 × *g* poly(ethyleneglycol) 6000 was added to a final concentration of 10% (mass/vol.). After centrifugation for 20 min at 20000 × *g*, E2p was precipitated from the supernatant with $MgCl_2$ (10–20 mM, depending on the protein concentration). After centrifugation for 15 min at 8000 × *g*, the pellet was suspended in 10 mM potassium phosphate buffer (pH 7.0), containing 0.1 mM $PhMeSO_2F$ and 10–20 mM EDTA (depending on the $MgCl_2$-concentration used for the precipitation), applied to a DEAE-Sephacel column and eluted with a KCl-gradient (0–500 mM) in 10 mM potassium phosphate buffer (pH 7.0), containing 0.5 mM EDTA and 0.1 mM $PhMeSO_2F$. The peak fraction was concentrated by ultrafiltration (Amicon YM 100) and applied to a Sephacryl-S400 column in standard buffer, containing only 0.5 mM EDTA. The peak fractions of this step were concentrated to a protein concentration of 5 mg/ml.

For the purification of the *A. vinelandii* E2p-mutant with one lipoyl domain (pAA100) and of *E. coli* E2p from *E. coli* TG2(pAW10) 30 g cells were suspended in standard buffer and disrupted using a Manton-Gaulin laboratory homogenizer at $6.2 \cdot 10^7$ Pa (9000 Psi). The following steps were performed as described for the wild type E2p from *A. vinelandii*.

The purification of the *A. vinelandii* E2p-mutants with partial or complete deletion of APA-4 (pAPE1 and pAPE2) is described in Ref. 23. The isolation of E2o from *A. vinelandii* is reported in Ref. 17.

Electron microscopy

E. coli TG2 cells, harbouring the recombinant plasmids pRA282, pAW10 or pEBC1, were grown as described above. After centrifugation of 10 ml culture for 10 min at 6000 × *g* the pellet was washed once in distilled water and subsequently suspended in 3% glutaraldehyde in 0.1 M sodiumcacodylate buffer (pH 7.2). After fixation the cells were dehydrated in a graded ethanol series, embedded in Lowicryl K_4M and polymerized by UV-light at −35°C [29]. Immunolabeling was performed on ultrathin sections with specific antisera against intact E2p (anti-E2p) or the catalytic domain (anti-cat) from *A. vinelandii* E2p by the protein A/gold method as described in Ref. 30. Prior to use, the antisera were adsorbed against *E. coli* TG2(pUC9).

Results

The effect of lysozyme on the solubilization of different E2-proteins expressed in E. coli TG2

The influence of either lysozyme treatment or sonication on the solubilization of different E2-proteins from *E. coli* TG2 cells is shown in Fig. 4, Table I and Table II. After lysozyme treatment of *E. coli* TG2 cells, containing only the plasmid pUC9 without insert (Fig. 4, lane 1L) only weak bands were detectable, whereas after sonication a typical cell free extract pattern was obtained (Fig. 4, lane 1S). After lysozyme-incubation of *E. coli* TG2 cells, expressing *A. vinelandii* wild type E2p or an E2p-mutant with two lipoyl domains, surprisingly around 50% of the E2p and many

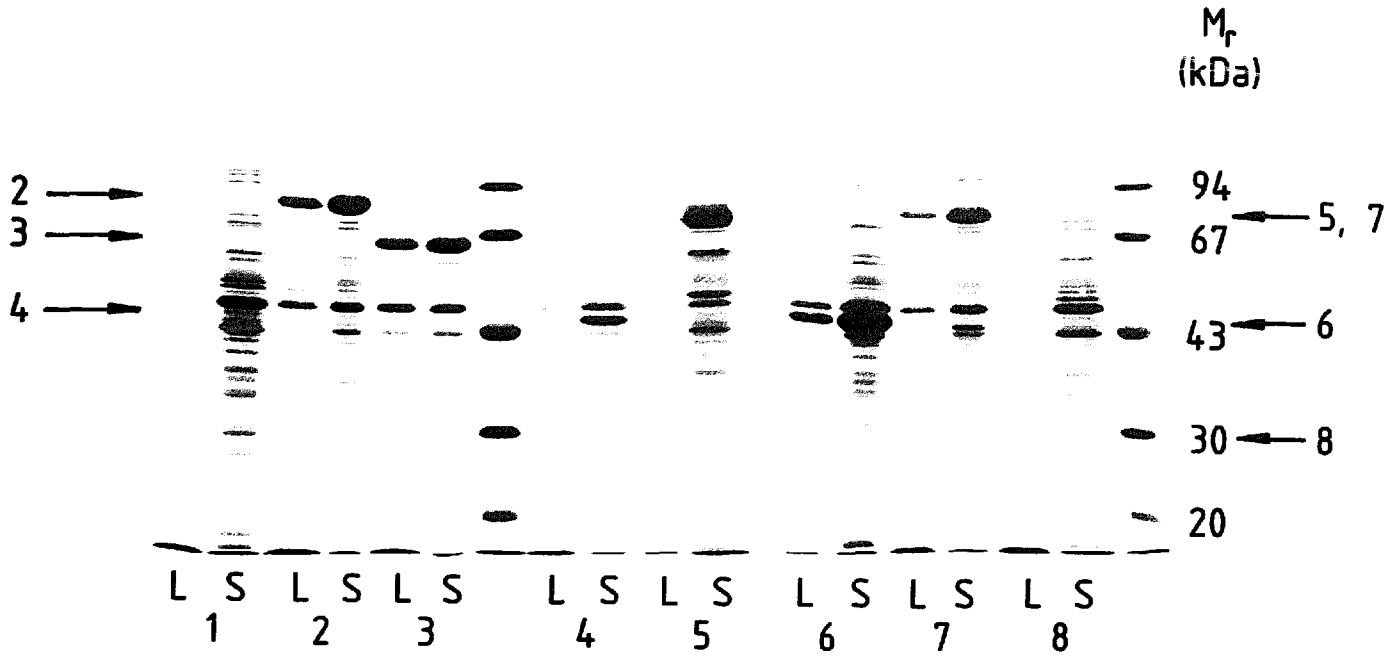

Fig. 4. SDS-gelelectrophoresis of cell free extracts from *E. coli* TG2 cultures expressing wild type and mutated E2 from *A. vinelandii* and *E. coli*. For the preparation of cell free extracts the cells were incubated with lysozyme (L) or sonicated (S). Arrows indicate the E2-proteins in the corresponding lanes. 1 – pUC9 without E2-insert as control; 2 – *A. vinelandii* wild type E2p (pRA282); 3 – *A. vinelandii* E2p with two lipoyl domains (pRA110); 4 – *A. vinelandii* E2p with one lipoyl domain (pAA100); 5 – *E. coli* wild type E2p (pAW10); 6 – E2 of the 2-oxoglutarate dehydrogenase complex from *A. vinelandii* (pAE2); 7 – *A. vinelandii* E2p with deletion of APA-4 (pAE2); 8 – *A. vinelandii* E2p binding and catalytic domain (pEBC1).

other cytoplasmic proteins were found in the soluble fraction (Fig. 4, lanes 2 and 3).

In order to investigate the intracellular localization of the E2p-proteins and the stability of the cell membrane during lysozyme incubation of *E. coli* TG2-cells, expressing *A. vinelandii* wild type E2p, the activities of periplasmic and cytoplasmic marker enzymes and of E2p were measured in the soluble fraction (Fig. 5). The activity of alkaline phosphatase as a periplasmic marker enzyme increased rapidly and all of the enzyme was solubilized within 20 min. The appearance of the cytoplasmic enzyme isocitrate dehydrogenase during lysozyme treatment occurred much more slowly and its activity stabilized between 1 and 2 h. The time-course of the E2p-activity is comparable with the pattern obtained for isocitrate dehydrogenase.

The amount of solubilized E2p after lysozyme incubation decreased with the deletion of lipoyl domains. 46% of the E2p-mutant with two lipoyl domains could be solubilized by lysozyme and only 10% of the E2p-mutant with one lipoyl domain was found in the lysozyme fraction (Table II). In contrast, the amount of solubilized dihydrolipoyltranssuccinylase (E2o), which contains only one lipoyl domain, in the lysozyme frac-

TABLE I

Enzyme activities of dihydrolipoyltransacetylase (E2p), isocitrate dehydrogenase (IDH), pyruvate dehydrogenase complex (PDC) and lipoamide dehydrogenase (E3) in cell-free extracts of E. coli TG2 cells (expressing different dihydrolipoyltransacetylases) after lysozyme treatment (60 min) or sonication 3·10 s)

E. coli TG2 containing only the plasmid pUC9 without insert is used as a control. The activities are given as the total amount of enzyme found in the fractions, which were prepared from 10 ml culture. For the preparation of the fractions see Materials and Methods. n.d., not detectable.

Expressed enzyme	Fraction	E2-activity (U)	IDH-activity (U)	PDC-activity (U)	E3-activity (U)
A. vinelandii	lysozyme	14.8	0.60	n.d.	0.23
wild type E2p	sonication	27.1	1.30	0.22	0.85
E. coli	lysozyme	0.2	0.08	n.d.	0.06
wild type E2p	sonication	11.5	1.93	0.38	1.86
A. vinelandii	lysozyme	0.4	0.07	n.d.	0.05
binding plus catalytic domain	sonication	7.6	2.32	0.33	1.26
pUC9	lysozyme	n.d.	n.d.	n.d.	n.d.
(without insert)	sonication	0.35	2.48	0.39	1.20

TABLE II

Enzyme activities of dihydrolipoyltransacetylase (E2p) in the cell-free extract of E. coli TG2 cells (expressing different E2p-mutants) after lysozyme treatment or sonication

The activities are given as the total amount of E2 found in the fraction, which was prepared from 10 ml culture.

Expressed E2p-mutant	E2-activity [U]	
	lysozyme fraction	sonicated fraction
A. vinelandii wild type E2 (pRA282)	14.8	27.1
A. vinelandii E2 with 2 lipoyl domains (pRA110)	10.2	22.1
A. vinelandii E2 with 1 lipoyl domain (pAA130)	1.4	13.4
A. vinelandii E2 with partial deletion of apa-4 (pAPE1)	10.1	24.6
A. vinelandii E2 with complete deletion of apa-4 (pAPE2)	7.4	19.5

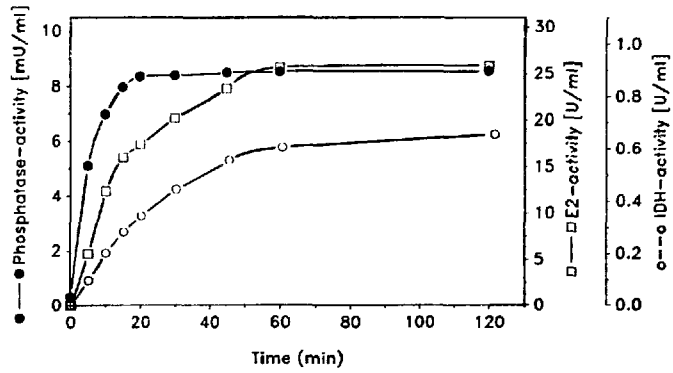

Fig. 5. Activities of alkaline phosphatase (filled circles), isocitrate dehydrogenase (IDH – open circles) and dihydrolipoyltransacetylase (open squares) in the cell-free extract of *E. coli* TG2 expressing *A. vinelandii* wild type E2p after lysozyme treatment. 100 ml culture (20 h) were centrifuged at 8000× *g* and the pellet suspended in 5 ml TEG-buffer. 100 µl lysozyme (50 mg/ml) were added to this suspension and incubated at 37°C. Aliquots were taken at different time points and activities of the enzymes were estimated in the supernatant after centrifugation at 11000× *g* (soluble fraction).

tion is relatively high (Fig. 4, lane 6). From the mutant with deletion of all three lipoyl domains only 5% could by solubilized after lysozyme-incubation (Table I).

A partial or complete deletion of the alanine- and proline-rich region between binding and catalytic domain of E2p (APA-4) is also influencing the amount of

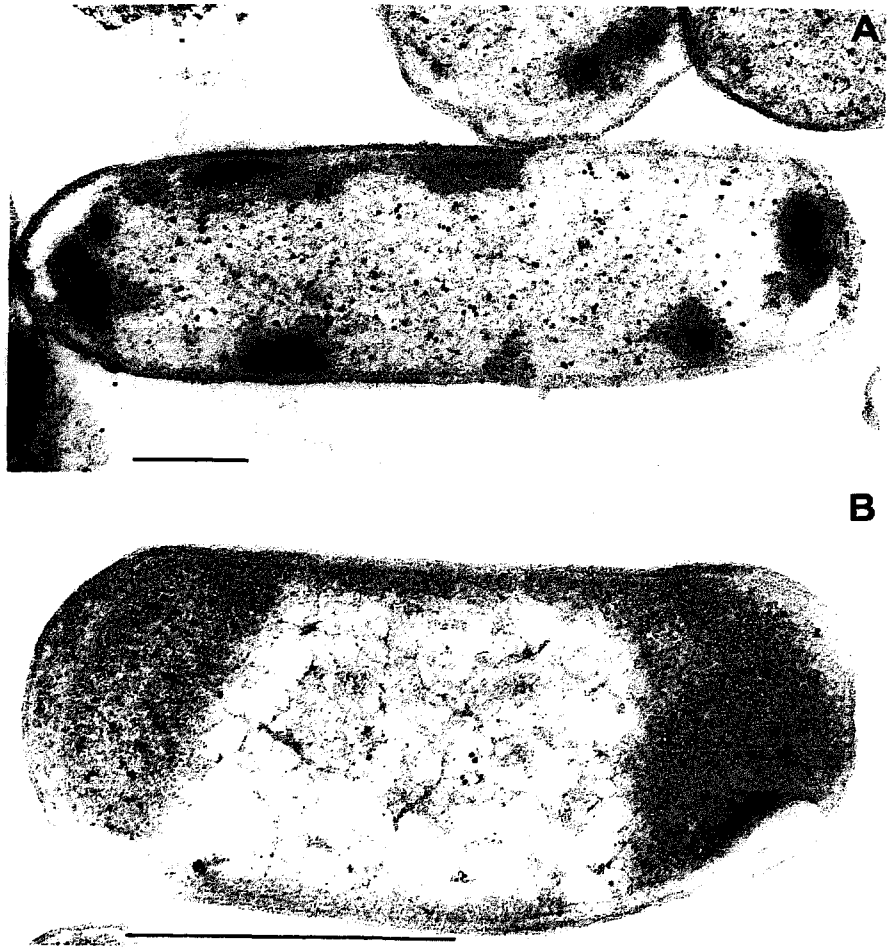

Fig. 6. Immunocytochemical experiments using protein A/gold and *A. vinelandii* E2p-antiserum on thin sections of *E. coli* TG2 cells, expressing different dihydrolipoyltransacetylases. (A) *A. vinelandii* wild type E2p (pRA282), anti-cat antiserum; (B) *A. vinelandii* E2p binding plus catalytic domain (pEBC1), anti-cat antiserum; (C, D) *E. coli* wild type E2p (pAW10), anti-E2 antiserum. The bar indicates 0.5 µm.

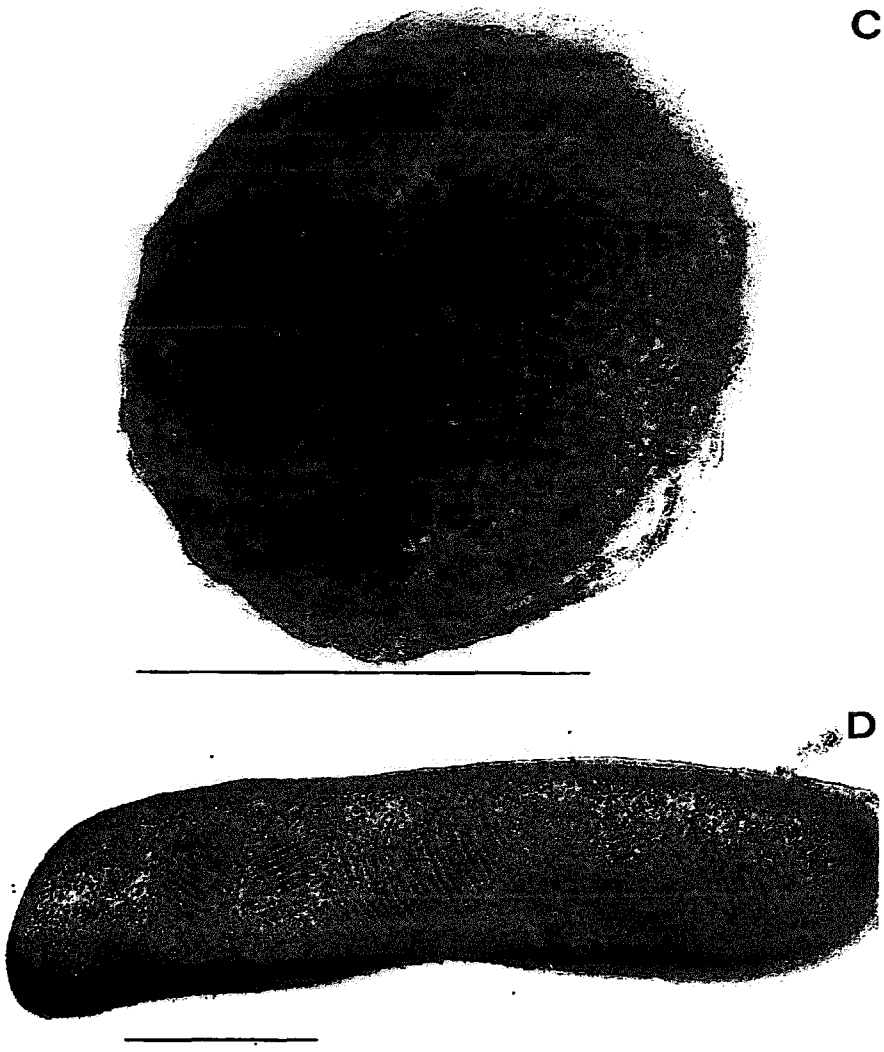

Fig. 6 (continued).

enzyme, which can be solubilized after lysozyme treatment. This amount decreased to 41% after the partial deletion of APA-4 (pAPE1), whereas after the complete deletion of APA-4 (pAPE2) only 27% of the total E2p-activity could be solubilized (Table II).

Lysozyme treatment of *E. coli* TG2 cells expressing *E. coli* wild type E2p did not result in a solubilization of E2p or other cytoplasmic proteins (Table I; Fig. 4, lane 5). After sonication less than half of the E2p-activity, which was found for *A. vinelandii* wild type E2p, could be detected (Table I), although the specific activity of *E. coli* wild type E2p (58 U/mg) is not significantly lower than that of *A. vinelandii* wild type E2p (64 U/mg).

The levels of isocitrate dehydrogenase present in the soluble fraction after lysozyme-incubation of *E. coli* TG2-cells, expressing different dihydrolipoyltransacetylases, may serve as a marker for the stability of the inner membrane (Table I). From *E. coli* TG2 cells, containing only the plasmid pUC9 without subcloned E2p-insert no isocitrate dehydrogenase could be solubilized by lysozyme. Only very low activities of isocitrate dehydrogenase were detectable in the lysozyme fraction of *E. coli* TG2 cells, expressing *E. coli* wild type E2p (pAW10) or the binding plus catalytic domain from *A. vinelandii* E2p (pEBC1), whereas 46% of the total enzyme activity were found in the lysozyme fraction of *E. coli* TG2 expressing *A. vinelandii* wild type E2p.

The relatively high activity of lipoamide dehydrogenase in the lysozyme fraction of *E. coli* TG2 expressing *A. vinelandii* wild type E2p is due to *E. coli* E3 which is bound on the expressed *A. vinelandii* E2p (Table I). The contaminating amount of E3 was always less than 2% of the E3, which can be bound to *A. vinelandii* wild type E2p within the complex. This E3 can easily be dissociated from E2p by gel-filtration in 2.5 M potassium bromide (pH 9.5). The lipoamide dehydrogenase activity in *E. coli* TG2 expressing *E. coli* wild type E2p (pAW10) is even 50% higher than observed in the control (pUC9); however, the activity of pyruvate dehydrogenase complex has not changed.

The results were independent on the buffer used. When, instead of TEG-buffer, an isotonic buffer containing 10 mM Tris-HCl (pH 8.0), 250 mM sucrose and 5 mM EDTA was used, identical results were obtained. In the absence of EDTA no E2p-activity and only 5–10% of the alkaline phosphatase activities were found in the supernatant after lysozyme treatment, indicating that the outer membrane stays mainly intact under these conditions.

Electron microscopy

Electron microscopical experiments were performed on *E. coli* TG2 cells expressing different dihydrolipoyltransacetylases from *A. vinelandii* and *E. coli* to visualize the intracellular localization of these proteins (Fig. 6).

The labeling patterns, obtained after immunocytochemical experiments on *A. vinelandii* wild type E2p with anti-E2p antiserum and protein A/gold, indicated that the protein is located randomly in the cytosol (Fig. 6A). Characteristic for these cells are electron dense regions, which were found at or near the cell membrane, but which showed no specific reaction with the antiserum. Labeling and/or electron dense spots were never observed in the periplasmic space. In contrast, most of the *A. vinelandii* E2p-mutant, which contains only the binding plus catalytic domain (pEBC1) was found in inclusion bodies located at both poles of the cell. The electron dense regions near the cell membrane, typical for *A. vinelandii* wild type E2p, were not observed (Fig. 6B).

Electron micrographs of *E. coli* TG2 cells, expressing *E. coli* wild type E2p are shown in Fig. 6C and D. The cytoplasm of the TG2 cells is occupied by highly ordered crystal-like structures in hexagonal array with a diameter of approx. 15 nm. Preliminary X-ray data indicate a diameter of 13.6 nm for the inner core structure (A. Mattevi, personal communication). Judged from the labeling patterns, the crystal-like structures represent aggregated E2p. Labeling is less intensive due to the fact that the anti-*A. vinelandii* E2p antiserum used in these experiments, showed a reduced cross reactivity to *E. coli* E2p as deduced from Western blots.

Purification of E2p expressed in E. coli TG2

The solubilizing effect of lysozyme for E2p, synthesized in *E. coli* TG2 cells was included in the standard scheme for the purification of wild type E2p from *A. vinelandii* and of mutants with at least two lipoyl domains and no deletions in APA-3 and APA-4. The results of a purification of *A. vinelandii* wild type E2p from *E. coli* TG2(pRA282) are shown in Fig. 7 and Table III. The total amount of E2p synthesized (12500 U), is comparable with the values observed previously [7]. 54% of the total E2p could be solubilized after incubation of the cells with lysozyme. The specific E2p-activity in the lysozyme fraction is twice as high as in an extract obtained after sonication. The contamination of the cell-free extract after lysozyme treatment with other proteins is in comparison to a cell-free extract obtained after sonication much lower (Figs. 4 and 7). The main advantage is that the extract is free from contamination with wild type *E. coli* complex.

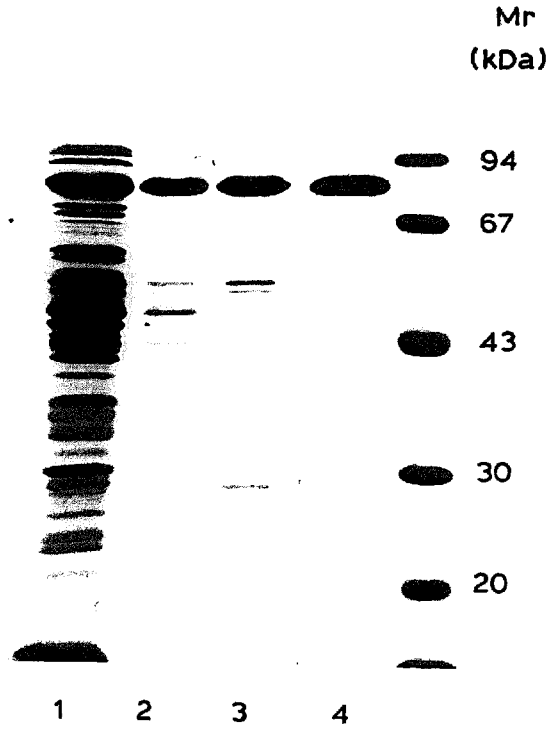

Fig. 7. Isolation of *A. vinelandii* wild type E2p from *E. coli* TG2 (pRA282). SDS-gel electrophoresis of cell-free extract after sonication (lane 1), cell free extract after incubation with lysozyme (lane 2), pool after DEAE-Sephacel chromatography (lane 3) and pool after Sephacryl S-400 gel filtration (lane 4).

TABLE III

Purification of A. vinelandii wild type E2p from E. coli TG2 (pRA282) after lysozyme incubation

The supernatant after lysozyme incubation and centrifugation at 14000 × *g* was used for the purification (crude extraxt – lysozyme). The pellet of the first centrifugation was resuspended in 100 ml standard buffer and the cells disrupted by sonication (crude extract – sonication).

Samples	Volume (ml)	Protein (mg)	Specific activity (U/mg)	Total units (U)	Yield (%)
Crude extract (sonication)	100	1834	3.2	5800	
Crude extract (lysozyme)	150	1030	6.5	6700	100
PEG/$MgCl_2$-precipitation	60	560	11.0	6180	92
DEAE-Sephacel	240	107	51.2	5480	82
Sephacryl S-400	110	78	64.1	5000	75

In contrast, *E. coli* wild type E2p and *A. vinelandii* E2p-mutants with less than 2 lipoyl domains could only be purified after disruption of cells by sonication or other mechanical treatments. The contamination with PDC must be carefully monitored during purification, but the large complex can be completely removed in the PEG/$MgCl_2$-precipitation step. From a 6 l culture of *E. coli* TG2 expressing *E. coli* wild type E2p 4500 U enzyme with a specific activity of 58 U/mg could be purified. As shown in Table I, the PDC-activity is not increased in *E. coli* TG2, expressing *E. coli* wild-type E2p, whereas the E3-activity is somewhat enhanced.

Discussion

Solubilization of overexpressed E2p-proteins from E. coli TG2

From the activity measurements of periplasmic and cytosolic marker enzymes during lysozyme incubation of *E. coli* TG2, expressing *A. vinelandii* wild type E2p and from the electron micrographs it can be concluded, that E2p is not localized in the periplasm. E2p is solubilized in the same time scale as the cytosolic marker enzyme isocitrate dehydrogenase, indicating that the bacterial inner membrane is at least partially permeabilized. This effect can only be explained by a change in the properties of the bacterial inner membrane, caused by the overexpression of E2p. We speculate, that the electron dense regions near the inner membrane of *E. coli* TG2-cells, expressing *A. vinelandii* E2p with 2 or 3 lipoyl domains, represent aggregated cytosolic proteins. These aggregations are probably caused by an excluded volume effect, comparable to the aggregating effects of poly(ethyleneglycol)-like components. The aggregated cytosolic proteins on or near the inner bacterial membrane could affect membrane integrity either by direct physical interactions and/or by indirect effects on the phospholipid metabolism. After degradation of the outer membrane by lysozyme, small proteins, like isocitrate dehydrogenase, or proteins which are expressed in the cells in enhanced levels (e.g. E2p) can diffuse through the permeable inner membrane. Large protein complexes, such as PDC, could not be detected in these fractions.

E2p from *A. vinelandii*, containing 2 or 3 lipoyl domains is highly soluble, in contrast to *A. vinelandii* E2p-mutants with one remaining lipoyl domain or with deletion of all three lipoyl domains. As indicated in Fig. 6B and C the latter mutated protein as well as *E. coli* wild type E2p aggregate in the cell and have therefore a much weaker effect on the dislocation of other cytoplasmic proteins. Therefore, the disturbing effect on membrane integrity in cells overexpressing these mutated proteins or *E. coli* wild type E2p was not observed.

The excluded volume effect, caused by overexpression of *A. vinelandii* wild type E2p or E2p-mutants with at least two lipoyl domains, is probably due to a combination of high solubility and special structural properties of the E2p-cubes, forming an extended network in the cytoplasmic space. The highly ordered aggregates found in cells overexpressing *E. coli* wild type E2p are indicative of this property.

Both, solubility and structural properties could be affected by the deletion mutants described here. The lipoyl domains are highly charged and prevent aggregation of *A. vinelandii* E2p. Stepwise deletion of these domains can thus explain the diminished lysozyme effect. On the other hand, reconstruction of E2p with 0, 1 or 2 lipoyl domains also requires mutations in the remaining apa-sequences (see Fig. 2). The effect on solubilization by lysozyme of E2p-proteins with a partial or complete deletion of the apa-sequence clearly points to an important role of the apa-sequences. A large variation exists between the apa-sequences of the complexes studied here, both in length and in the content of hydrophobic/hydrophilic residues, whereas a high similarity exists between the lipoyl domain sequences.

It is of interest to note here a relation that could exist with biliary cirrhosis [31,32]. In this autoimmune disease antibodies are made against E2, normally localized in the mitochondria. If for some reason the concentration of E2 is raised in the cytosol, this could lead to cell leakage and in this way to the formation of auto-antibodies against E2.

E. coli wild type E2p shows in its primary structure an amino acid sequence identity of 50% with *A. vinelandii* E2p [3]. The first three APA-sequences of *E. coli* E2p are less hydrophilic whereas APA-4 is extremely short and contains only one charged residue [9]. These differences might explain the high rate of aggregation of *E. coli* E2p and thus, the lack of any solubilization after lysozyme treatment.

Purification of E2p from E. coli TG2

For investigations on *A. vinelandii* PDC, reconstitutions of the complex from its individual components have many advantages in comparison with the expression and purification of the whole complex from PDC-deletion mutants as described for example in Ref. 33. The peripheral components E1p and E3 can be easily purified and the expression of *A. vinelandii* E2p and even of *E. coli* E2p in *E. coli* TG2 cells is much higher, than described for PDC-deletion mutants [33]. Pyruvate dehydrogenase complexes with high specific activities (around 50 U/mg E2) or subcomplexes containing only E1 or E3 and different E2p-mutants were reconstituted from the individual components and used for investigations of the binding properties and the catalytic mechanism of the complex [11].

The enhanced levels of E3 in *E. coli* TG2 cells, expressing *E. coli* wild type E2p are probably due to regulation of *E. coli* E3-synthesis by an own promoter [24], which is necessary to satisfy the E3-requirements of the *E. coli* 2-oxoglutarate dehydrogenase complex [34]. The *E. coli* genes for E1 and E2p are probably controlled together by a promoter upstream of the start codon for E1p [4]. Therefore, no extra E1p is synthesized from *E. coli* TG2 cells, expressing *E. coli* wild type E2p and no increase of the PDC-activity was detectable. On the other hand, the purification of mutated *E. coli* E2p from TG2-cells will be more complicated, because of a contamination by chromosomal wild type E2p.

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